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LITTON BIONETICS INC

Document Title

MUTAGENICITY EVALUATION OF LIQUID PREPOLYMER 11664-97 IN THE MOUSE LYMPHOMA FORWARD MUTATION ASSAY (FINAL REPORT) WITH COVER LETTER DATED 011591

Chemical Category

4,4-DIPHENYLMETHANE DIISOCYANATE (101-68-8)

CONTAINS NO CBI

GRACE - 86-910000649

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91 JAN 24 AM 9:44

January 16, 1991

Environmental Protection Agency
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Washington, D.C. 20460

Attn: Health and Safety Reporting Rule (Notification/Reporting)

Please find attached 8(d) health and safety reports for mixtures processed containing toluene diisocyanate (CAS #26471-62-5), 4,4-Diphenylmethane diisocyanate (CAS #101-68-8) and 1,6-Diisocyanatohexane (CAS #822-06-0). Grace is submitting these reports for late filing since their submittal may have been subject to the isocyanates 10-year call-in of June 1, 1987.

We have reason to believe that some of these reports may have previously been submitted to EPA as attachments to PMN submissions. However, Grace is filing them as a precautionary measure to insure EPA's receipt.

These reports are being submitted for:

W. R. Grace & Co.-Conn.
Washington Research Center
7379 Route 32
Columbia, MD 21044

Sincerely,

J. W. Raksis

A:\JR91-013/1w

Attachments - 20

86910000638

GENETICS ASSAY NO. 5919

LBI SAFETY NO. 7237

4,4-Diphenylmethane diisocyanate
101-68-8

MUTAGENICITY EVALUATION OF LIQUID PREPOLYMER 11664-97 IN THE MOUSE LYMPHOMA FORWARD MUTATION ASSAY

FINAL REPORT

SUBMITTED TO:

W.R. GRACE & CO.
7379 ROUTE 32
COLUMBIA, MARYLAND 21044

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895

LBI PROJECT NO. 20989

REPORT DATE: DECEMBER, 1981

PREFACE

This report contains a summary of the data compiled during the evaluation of the test compound. The report is organized to present the results in a concise and easily interpretable manner. The first part contains Items I-IX. Items I-IV provide sponsor and test article identification information, type of assay, and the protocol reference number. Item V provides the initiation and completion dates of the study. Item VI identifies the supervisory personnel. Item VII indicates the tables and/or figures containing the test results. The interpretation of the results is in Item VIII. Item IX provides the conclusion and evaluation.

The second part of the report describes the study design, which includes the materials and procedures employed in conducting the assay. This part of the report also contains evaluation criteria used by the study director, and any appendices.

All test and control results presented in this report are supported by raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895.

Copies of the raw data will be supplied to the sponsor upon request.

The described study was performed in accordance with Good Laboratory Practice regulations except if noted to the contrary. To the best of the signer's knowledge there were no significant deviations from the Good Laboratory Practice regulations which affected the quality or integrity of the study.

I. SPONSOR: W.R. Grace & Co.

II. MATERIAL (TEST COMPOUND): GENETICS ASSAY NO.: 5919

A. Identification: Liquid prepolymer 11664-97 HYPOL 4100

B. Date Received: September 24, 1981

C. Physical Description: Viscous, pale-yellow liquid

III. TYPE OF ASSAY: Mouse Lymphoma Forward Mutation Assay

IV. ASSAY DESIGN NUMBER: 431

V. STUDY DATES:

A. Initiation: September 28, 1981

B. Completion: October 30, 1981

VI. SUPERVISORY PERSONNEL:

A. Study Director: Maria A. Cifone, Ph.D.

B. Laboratory Supervisor: Jane Fisher

VII. RESULTS:

The data are presented in Table 1 on page 4.

VIII. INTERPRETATION OF RESULTS:

The test material, liquid prepolymer 11664-97, formed a white solid in a clear colorless liquid with water at 100 µl/ml but formed a clear colorless liquid in dimethylsulfoxide (DMSO) at the same concentration. DMSO was chosen as the solvent and, just prior to testing, stocks were prepared by performing serial dilutions in DMSO. The assay was initiated by performing final 1:100 dilutions of the stock solutions into medium containing the cells. The test material appeared soluble up to 31.3 nl/ml without activation and 0.977 nl/ml in the presence of rat liver S9 microsomal activation mix. Higher concentrations appeared cloudy and contained a white precipitate that increased with increasing concentrations of test material (up to the highest concentration tested, 1000 nl/ml).

One trial of the mutation assay was initiated and the results are presented in Table 1.

Under nonactivation conditions, no evidence for mutagenic activity was obtained. The mutant frequencies in the cultures exposed to concentrations of test material from 31.3 nl/ml to 500 nl/ml ranged from 12.1×10^{-6} to 20.4×10^{-6}. A mutant frequency exceeding 27.8×10^{-6} was considered to be the minimum criterion for indicating mutagenesis by a given treatment in this assay. The relative growths of the assayed treatments ranged from

VIII. INTERPRETATION OF RESULTS: (continued)

78.7% to 21.4% which demonstrates moderate to low toxicity. The next increment in concentration to 1000 nl/ml was completely lethal to the cells (data not shown). These results showed that the test material was not detectably mutagenic under nonactivation conditions for concentrations that closely approached a level causing excessive toxicity.

In the presence of S9 microsomal activation mix, the test material caused significant increases in the mutant frequency. The minimum criterion for indicating mutagenesis in this portion of the assay was a mutant frequency exceeding 51.6 x 10^{-6}. This value was just exceeded in the culture exposed to 125 nl/ml of test material, which resulted in moderately high toxicity (23.2% relative growth). The 250 nl/ml treatment was very toxic (15.3% relative growth) and induced a 4.4-fold increase in mutant frequency over the average negative control value. The next highest concentration of test material (500 nl/ml) was also highly toxic (6.1% relative growth) and induced a mutant frequency that was 5.6-fold above the background. Thus, the observed response appeared to be dose-related and was interpreted to mean that the test material was converted by the metabolic activation system to one or more mutagenic products.

The average cloning efficiencies for the solvent and untreated negative controls varied from 109.2% without activation to 105.6% with activation which demonstrated excellent cloning conditions for the assays. The negative control mutant frequencies were all in the normal range and the positive control compounds yielded normal mutant frequencies that were greatly in excess of the background.

IX. CONCLUSIONS:

The test material, Liquid Prepolymer 11664-97, induced dose-related increases in the mutant frequency at the TK locus in L5178Y mouse lymphoma cells only in the presence of rat liver S9 microsomal activation. Without activation, concentrations up to 500 nl/ml demonstrated moderately high toxicity without being detectably mutagenic, and 1000 nl/ml was completely lethal. With activation, mutagenesis was detected at 125 nl/ml and increased in a dose-dependent manner at 250 nl/ml and 500 nl/ml. The increases in the mutant frequency ranged from 2.1-fold to 5.6-fold above the background (average of solvent and untreated control mutant frequencies). The test material is therefore considered to be active in the Mouse Lymphoma Forward Mutation Assay with microsomal activation.

Submitted by:

Study Director

Maria A. Cifone 12-9-81

Maria A. Cifone, Ph.D. Date
Cell Biologist
Department of
Molecular Toxicology

Reviewed by:

David J. Brusick 12/9/81

David J. Brusick, Ph.D. Date
Director
Department of
Molecular Toxicology

4. SUMMARY OF MOUSE LYMPHOMA (L5178Y) RESULTS TABLE 1

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: LIQUID PREPOLYMER 11664-97 HYPOL 4100
B. LBI CODE #: 5919
C. SOLVENT: DMSO
D. TEST DATE: 10/19/81

TEST	S-9 SOURCE	S-9 TISSUE	DAILY COUNTS (CELLS/ML X 10E5) 1	2	3	RELATIVE SUSPENSION GROWTH (% OF CONTROL)	TOTAL MUTANT CLONES	TOTAL VIABLE CLONES	RELATIVE CLONING EFFICIENCY (% OF CONTROL)	PERCENT RELATIVE GROWTH*	MUTANT FREQUENCY**
NONACTIVATION											
SOLVENT CONTROL	---	---	13.7	10.0		100.0	32.0	289.0	100.0	100.0	11.1
SOLVENT CONTROL	---	---	16.5	13.9		100.0	37.0	394.0	100.0	100.0	9.4
UNTREATED CONTROL	---	---	13.8	14.5		109.2	45.0	300.0	87.8	96.0	15.0
EMS .5 UL/ML	---	---	9.7	7.0		37.1	174.0	53.0	15.5	5.8	328.3
TEST COMPOUND											
31.300 NL/ML	---	---	13.5	13.6		100.2	36.0	268.0	78.5	78.7	13.4
62.500 NL/ML	---	---	11.5	10.2		64.0	32.0	265.0	77.6	49.7	12.1
125.000 NL/ML	---	---	13.3	10.9		79.1	45.0	289.0	84.6	67.0	15.6
250.000 NL/ML	---	---	14.0	7.6		58.1	36.0	182.0	53.3	31.0	19.8
500.000 NL/ML	---	---	11.0	8.0		48.0	31.0	152.0	44.5	21.4	20.4
ACTIVATION											
SOLVENT CONTROL	RAT	LIVER	10.4	13.3		100.0	107.0	372.0	100.0	100.0	28.8
SOLVENT CONTROL	RAT	LIVER	10.0	10.4		100.0	79.0	283.0	100.0	100.0	27.9
UNTREATED CONTROL	RAT	LIVER	11.0	13.6		123.5	78.0	295.0	90.1	111.2	26.4
DMN .3 UL/ML	RAT	LIVER	7.0	7.4		42.8	79.0	34.0	10.4	4.4	232.4
TEST COMPOUND											
31.300 NL/ML	RAT	LIVER	10.8	9.0		80.2	53.0	201.0	61.4	49.2	26.4
62.500 NL/ML	RAT	LIVER	7.8	8.2		52.8	65.0	135.0	41.2	21.8	48.1
125.000 NL/ML	RAT	LIVER	8.6	6.6		46.8	96.0	162.0	49.5	23.2	59.3
250.000 NL/ML	RAT	LIVER	6.7	7.5		41.5	146.0	121.0	36.9	15.3	120.7
500.000 NL/ML	RAT	LIVER	4.9	6.3		23.4	132.0	85.0	26.0	6.1	155.3

* (RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY) / 100
** THE RATIO OF CELLS SEEDED FOR MUTANT SELECTION TO CELLS SEEDED FOR CLONING EFFICIENCY IS 10E+4.
THEREFORE THE MUTANT FREQUENCY IS: (TOTAL MUTANT CLONES/TOTAL VIABLE CLONES)*10E-4.
THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10E-6.

ASSAY DESIGN NO. 431

CHANGE SHEET

1. The dosage selection portion of the Assay Design (Part 4.A) was not performed as a separate, preliminary cytotoxicity test as was usually done for previous studies. Instead, dose selection became an integral part of the mutation assay by the use of the range of concentrations normally employed in the preliminary test. More often than not, a suitable number of treatments will be available for mutant analysis, but if not, a second trial with an adjusted dose range for either activation test condition would then be initiated. This procedure appears to be more efficient in time and materials and is under serious consideration of becoming standard procedure.

2. The concentration of Fischer 344 Aroclor-induced rat liver S9 microsomal activation mix was reduced from 0.5 ml per 10 ml assay to 0.3 ml per 10 ml. Due to the variable nature of different batches of rat liver S9 activation mix, adjustment of the concentration of S9 is sometimes necessary. The reduced concentration resulted in positive control mutant frequencies that were in keeping with the historical data.

ASSAY DESIGN (NO. 431)

1. OBJECTIVE

The objective of this study is to evaluate the test material for its ability to induce forward mutation in the L5178Y TK+/- mouse lymphoma cell line, as assessed by colony growth in the presence of 5-bromo-2'-deoxyuridine (BrdU) or 5-trifluorothymidine (TFT).

2. RATIONALE

Thymidine kinase (TK) is a cellular enzyme that allows cells to salvage thymidine from the surrounding medium for use in DNA synthesis. If a thymidine analog such as BrdU is included in the growth medium, the analog will be phosphorylated via the TK pathway and be incorporated into DNA, eventually resulting in cellular death. Cells which are heterozygous at the TK locus (TK+/-) may undergo a single step forward mutation to the TK -/- genotype in which little or no TK activity remains. Such mutants are as viable as the heterozygotes in normal medium because DNA synthesis proceeds by de novo synthetic pathways that do not involve thymidine as an intermediate. The basis for selection of the TK-/- mutants is the lack of any ability to utilize toxic analogs of thymidine, which enables only the TK-/- mutants to grow in the presence of BrdU. Cells which grow to form colonies in the presence of BrdU are therefore assumed to have mutated, either spontaneously or by the action of a test substance, to the TK-/- genotype.

3. MATERIALS

A. Indicator Cells

The mouse lymphoma cell line, L5178Y TK+/-, used in this assay is derived from the Fischer L5178Y line of Dr. Donald Clive. Stocks are maintained in liquid nitrogen and laboratory cultures are periodically checked for the absence of mycoplasma contamination by culturing methods. To reduce the negative control frequency (spontaneous frequency) of TK-/- mutants to as low level as possible, cell cultures are exposed to conditions which select against the TK-/- phenotype (exposure to methotrexate) and are then returned to normal growth medium for three or more days before use.

B. Media

The cells are maintained in Fischer's mouse leukemia medium supplemented with L-glutamine, sodium pyruvate, and horse serum (10% by volume). Cloning medium consists of the preceding growth medium with the addition of agar to a final concentration of 0.35% to achieve a semisolid state. Selection medium is cloning medium containing 100 µg/ml of BrdU or 3 µg/ml of TFT.

3. MATERIALS (continued)

C. Control Compounds

1. Negative Controls

A negative control consisting of assay procedures performed on untreated cells is performed in all cases. If the test compound is not soluble in growth medium, an organic solvent (normally DMSO) is used; the final concentration of solvent in the growth medium will be 1% or less. Cells exposed to solvent in the medium are also assayed as the solvent negative control to determine any effects on survival or mutation caused by the solvent alone. For test substances assayed with activation, the untreated and solvent negative controls will include the activation mixture.

2. Positive Controls

Ethylmethane sulfonate (EMS) is highly mutagenic via alkylation of cellular DNA and will be used at 0.5 µl/ml as a positive control for nonactivation studies.

Dimethylnitrosamine (DMN) requires metabolic activation by microsomal enzymes to become mutagenic and will be used at 0.3 µl/ml as a positive control for assays performed with activation.

D. Sample Forms

Solid materials are dissolved in growth medium, if possible, or in DMSO, unless another solvent is requested. Liquids are tested by direct addition to the test system at predetermined concentrations or following dilution in a suitable solvent.

4. EXPERIMENTAL DESIGN

A. Dosage Selection (Cytotoxicity testing)

The solubility of the test chemical in growth medium and/or DMSO is first determined. Then a wide range of chemical concentrations is tested for cytotoxicity, starting with a maximum applied dose of 10 mg/ml for test chemicals soluble in media or 1 mg/ml for solutions in organic solvents. After an exposure time of four hours, the cells are washed and a viable cell count is obtained the next day. Relative cytotoxicities expressed as the reduction in growth compared to the growth of untreated cells are used to select seven to ten doses that cover the range from 0 to 50-90% reduction in 24-hour growth. These selected doses are subsequently applied to cell cultures prepared for mutagenicity testing, but only four or five of the doses will be carried through the mutant selection process. This procedure compensates for daily variations in cellular cytotoxicity and ensures the choice of four or five doses spaced from 0 to 50-90% reduction in cell growth.

B. Mutagenicity Testing

1. Nonactivation Assay

The procedure used is based on that reported by Clive and Spector (1975) and is summarized as follows. Cultures exposed to the test chemical for four hours at the preselected doses are washed and placed in growth medium for two or three days to allow recovery, growth and expression of the induced TK-/- phenotype. Cell counts are determined daily and appropriate dilutions are made to allow optimal growth rates.

At the end of the expression period, 3×10^6 cells for each selected dose are seeded in soft agar plates with selection medium and resistant (mutant) colonies are counted after 10 days incubation. To determine the actual number of cells capable of forming colonies, a portion of the cell suspension is also cloned in normal medium (nonselective). The ratio of resistant colonies to total viable cell number is the mutant frequency.

A detailed flow diagram for the mutation assay is provided in Figure 1.

2. Activation Assay

The activation assay can be run concurrently with the nonactivation assay. The only difference is the addition of the S9 fraction of rat liver homogenate and necessary cofactors (CORE) during the four-hour treatment period. CORE consists of NADP (sodium salt) and isocitric acid. The final concentrations of the activation system components in the cell suspension are: 2.4 mg NADP/ml; 4.5 mg isocitric acid/ml; and 50 µl S9/ml.

3. S9 Homogenate

A 9,000 x g supernatant prepared from Fischer 344 adult male rat liver induced by Aroclor 1254 (described by Ames et al., 1975) is purchased from Bionetics Laboratory Products, Litton Bionetics, Inc. and used in this assay.

5. REPORT

The screened doses, cell counts, and mutant and viable colony counts will be entered into a computer program. The results are analyzed and printed.

The suspension growth of each culture is calculated as (Day 1 Cell Count/3) x (Day 2 Cell Count/3) x (Day 3 Cell Count/3) when the cultures are split back to 3 x 10^5 cells/ml after the daily count. If the cell count is less than 4 x 10^5 cells/ml, the culture is not split back and the cell count is substituted for 3 in the denominator of the next daily count. In most assays, 3-day expressions are not used, so only the first two factors in the preceding calculation are used. The suspension growth is calculated for each solvent control and then averaged. Relative suspension growth values are derived by dividing the suspension growth values by the average solvent control value and multiplying by 100%.

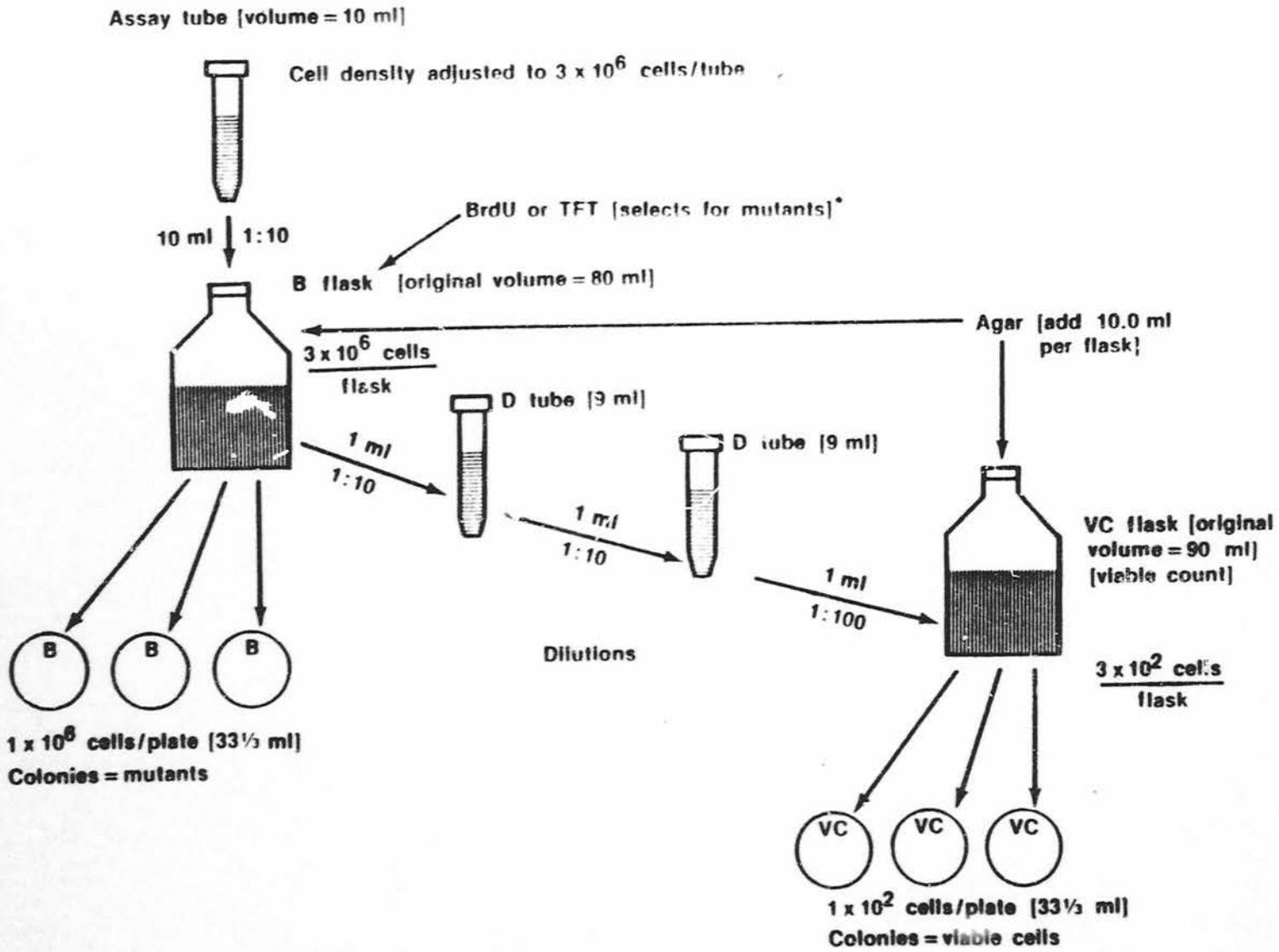
The average cloning efficiency for the negative controls in an assay is the average number of viable colonies for the solvent and untreated controls, divided by 300 and multiplied by 100%. In the computer tables, the cloning efficiency of each culture is expressed relative to the average solvent control cloning efficiency. Whenever the number of cells seeded for viable colony counts differs from 300, the computer calculation of the relative cloning efficiency is adjusted by the factor (300/cells seeded).

A percent relative growth value is calculated as (relative suspension growth) x (relative cloning efficiency/100). Corrected values for the relative cloning efficiency are used in the cases where the number of cells seeded for viable colonies differs from 300.

The mutant frequency is calculated as the ratio of mutant colonies to viable colonies times 10^{-4}. This calculation is unaffected by changes in the number of cells seeded for viable count because the number of cells seeded for mutant selection is changed by the same factor. Thus, as an example, if 250 cells are seeded for viable count, 2.5 x 10^5 cells are seeded for mutant selection· the 10^{-4} factor remains constant.

6. REFERENCE

Clive, D. and Spector, J.F.S.: Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutation Res., 31:17-29. 1975.



*Added after removal of 1 ml for viable count dilutions.

FIGURE 1. LYMPHOMA CLONING FLOW CHART

ASSAY ACCEPTANCE CRITERIA

An assay will normally be considered acceptable for evaluation of the test results only if all of the criteria given below are satisfied. The activation and nonactivation portions of the mutation assays are usually performed concurrently, but each portion is in fact an independent assay with its own positive and negative controls. The activation or nonactivation assays will be repeated independently, as needed, to satisfy the acceptance and evaluation criteria.

1) The average absolute cloning efficiency of the negative controls (average of the solvent and untreated controls) should be between 70% and 130%. A value greater than 100% is possible because of errors in cell counts (usually ± 10%) and cell division during unavoidable delays between the counting and cloning of many cell cultures. Cloning efficiencies below 70% do not necessarily indicate substandard culture conditions or unhealthy cells. Assay variables can lead to artificially low cloning efficiencies in the range of 50% to 70% and still yield internally consistent and valid results. Assays with cloning efficiencies in this range are conditionally acceptable and dependent on the scientific judgement of the study director. All assays below 50% cloning efficiency are unacceptable.

2) The solvent and untreated negative controls normally have the same growth rates and cloning efficiencies within experimental error. An unusual effect by the solvent therefore indicates an abnormal cell state or excessive amount of solvent in the growth medium. An assay will be unacceptable if the average percent relative growth of the solvent controls is less than about 70% of the untreated control value.

3) The minimum acceptable value for the suspension growth of the average negative control (average of the solvent and untreated control values) for two days is 8.0. Lower values will render an assay unacceptable for evaluation because of the high frequency of unreliable measurements for both the induced mutant frequency and toxicity of a given treatment. The value of 8 corresponds to three population doublings over the 2-day expression period. The most desirable range for the negative control suspension growth is 12 to 25, since the cells are capable of a 5-fold increase in number under optimal growth conditions for 24 hours.

4) The background mutant frequency (average frequency of the solvent and untreated negative controls) is calculated separately for concurrent activation and nonactivation assays, even though the same population of cells is used for each assay. The activation negative controls contain the S9 activation mix and typically have a somewhat higher mutant frequency than the nonactivation negative controls. For both conditions, the normal range of background frequencies for assays performed with different cell stocks is 5×10^{-6} to 50×10^{-6}. Assays with backgrounds outside this range are not necessarily invalid but will not be used as primary evidence for the evaluation of a test material. These assays can provide supporting evidence.

5) A positive control is included with each assay to provide confidence in the procedures used to detect mutagenic activity. The normal range of mutant frequencies induced by 0.5 µl/ml EMS (nonactivation assay) is 300 to 800 x 10^{-6}; for 0.3 µl/ml DMN (activation assay) the normal range is 200 to 800 x 10^{-6}. The concurrent background frequencies have been subtracted from these values. These ranges are broad primarily because the effective treatment with these agents is variable between assays. An assay will be acceptable in the absence of a positive control (loss due to contamination or technical error) only if the test material clearly shows mutagenic activity as described in the evaluation criteria. If the test material appears to have no or only weak mutagenic activity, an acceptable assay must have a positive control mutant frequency above the lower limits of the normal range. Assays in which the normal range is exceeded may require further interpretation by the study director.

6) For test materials with little or no mutagenic activity, an assay must include applied concentrations that reduce the suspension growth to 5% to 10% of the average solvent control or reach the maximum applied concentrations given in the evaluation criteria. Suspension growth is a combined measure of cell death and reduced growth rates. A 5% relative suspension growth therefore could correspond to 90% killing followed by growth of the survivors at one-half the normal rate for one day and normal growth for the second day. At the other extreme, this condition could be obtained by no killing and complete inhibition of growth for two days. A reasonable limit to testing for the presence of mutagenic action is about 80% to 90% killing of cells. Because of the uncertainty in the actual lethality of treatment in the assay and the fact that mutant frequencies increase as a function of lethality, an acceptable assay for the lack of mutagenic activity must extend to the 5% to 10% relative suspension growth range. There is no maximum toxicity requirement for test materials which clearly show mutagenic activity.

7) An experimental treatment that results in fewer than 2.5 x 10^6 cells by the end of the two-day growth period will not be cloned for mutant analysis.

8) An experimental mutant frequency will be considered acceptable for evaluation only if the relative cloning efficiency is 10% or greater and the total number of viable clones exceeds about 20. These limits avoid problems with the statistical distribution of scoreable colonies among dishes and allows factors no larger than 10 in the adjustment of the observed number of mutant clones to a unit number of cells (10^6) able to form colonies.

9) Mutant frequencies are normally derived from sets of three dishes for both the mutant colony count and the viable colony count. In order to allow for contamination losses, an acceptable mutant frequency can be calculated from a minimum of two dishes per set if the colony numbers in the two dishes differ by no more than about 3-fold.

10) The mutant frequencies for five treated cultures are normally determined in each assay. A required number of different concentrations cannot be explicitly stated, although a minimum of three analyzed cultures is considered necessary under the most favorable test conditions to accept a single assay for evaluation of the test material.

ASSAY EVALUATION CRITERIA

Mutation assays are initiated by exposing cell cultures to a range of concentrations of test material that is expected, on the basis of preliminary toxicity studies, to span the cellular responses of no observed toxicity to growth to complete lethality within 24 hours of treatment. Then five dose levels are usually selected for completion of the mutation assay. The doses are selected to cover a range of toxicities to growth with emphasis on the most toxic doses. An assay may need to be repeated with different concentrations in order to properly evaluate a test material.

The minimum condition considered necessary to demonstrate mutagenesis for any given treatment is a mutant frequency that exceeds 150% of the concurrent background frequency by at least 10×10^{-6}. The background frequency is defined as the average mutant frequency of the solvent and untreated negative controls. The minimum increase is based on extensive experience which indicates that assay variability increases with higher backgrounds and the calculated minimum increase as defined above is often a repeatable result; statistical analysis for the confidence limits is not yet available.

The observation of a mutant frequency that meets the minimum criterion for a single treated culture within a range of assayed concentrations is not sufficient evidence to evaluate a test material as a mutagen. The following test results must be obtained to reach this conclusion for either activation or nonactivation conditions:

- A dose-related or toxicity-related increase in mutant frequency should be observed. It is desirable to obtain this relation for at least three doses, but this depends on the concentration steps chosen for the assay and the toxicity at which mutagenic activity appears.

- An increase in mutant frequency may be followed by only small or no further increases at higher concentrations or toxicities. However, a decrease in mutant frequency to values below the minimum criterion is not acceptable in a single assay for classifying the test material as a mutagen. If the mutagenic activity at lower concentrations or toxicities was large, a repeat assay will be performed to confirm the mutagenic activity.

- If an increase of about two times the minimum criterion or greater is observed for a single dose near the highest testable toxicity, as defined in the Assay Acceptance Criteria, the test material will be considered mutagenic. Smaller increases at a single dose near the highest testable toxicity will require confirmation by a repeat assay.

- For some test materials, the correlation between toxicity and applied concentration is poor. The proportion of the applied material that effectively interacts with the cells to cause genetic alterations is not always repeatable or under control. Conversely, measurable changes in the frequency of induced mutants may occur with concentration changes that cause only small changes in observable toxicity. Therefore, either parameter, applied concentration or toxicity (percent relative growth), can be used to establish whether the mutagenic activity is related to an increase in effective treatment. A negative correlation with dose is acceptable only if a positive correlation with toxicity exists. An apparent increase in mutagenic activity as a function of decreasing toxicity is not acceptable evidence for mutagenicity.

A test material will be evaluated as nonmutagenic in a single assay only if the minimum increase in mutant frequency is not observed for a range of applied concentrations that extends to toxicity causing 5% to 10% relative suspension growth. If the test material is relatively nontoxic, the maximum applied concentrations will normally be 10 mg/ml (or 10 µl/ml) for water-soluble materials or 1 mg/ml (or 1 µl/ml) for materials in organic solvents. If a repeat assay does not confirm an earlier, minimal response, as discussed above, the test material will be evaluated as nonmutagenic in this assay system.

The ASSAY ACCEPTANCE AND EVALUATION CRITERIA are presented to acquaint the sponsor with the considerations used by the study director to determine assay validity and the mutagenic activity of the test material. This presentation may not encompass all test situations, and the study director may use other criteria, especially when data from several repeat assays are available, to arrive at a conclusion. The report will provide the reasoning involved when departures from the above descriptions occur.

Q.A. Inspection Statement
(reference 21 CFR 58.35(b)(7))

PROJECT 20989 LBI Assay No. 5919

TYPE of STUDY Mouse Lymphoma assay

This final study report was reviewed by the LBI Quality Assurance Unit on 12.9.81. A report of findings was submitted to the Study Director and to Management on 12.9.81.

The short-term nature of this study precluded inspection while it was in process. The Quality Assurance Unit inspects an in-process study of this type approximately once per month to assure that no significant problems exist that are likely to affect the integrity of this type of study.

Marshall S. Hyman
Auditor, Quality Assurance Unit

Litton BIONETICS

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